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ScienceDirect
Veterinary Parasitology 152 (2008) 8–15
veterinary parasitology
www.elsevier.com/locate/vetpar

Modest genetic differentiation among North American populations of *Sarcocystis neurona* may reflect expansion in its geographic range

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Received 1 November 2007; received in revised form 4 December 2007; accepted 10 December 2007

Abstract

Sarcocystis neurona is an important cause of neurological disease in horses (equine protozoal myeloencephalitis, EPM) and sea otters in the United States. In addition, EPM-like disease has been diagnosed in several other land and marine mammals. Opossums are its only definitive hosts. Little genetic diversity among isolates of *S. neurona* from different hosts has been reported. Here, we used 11 microsatellites to characterize *S. neurona* DNA isolated from natural infections in 22 sea otters (*Enhydra lutris*) from California and Washington and in 11 raccoons (*Procyon lotor*) and 1 striped skunk (*Mephitis mephitis*) from Wisconsin. By jointly analyzing these 34 isolates with 26 isolates previously reported, we determined that geographic barriers may limit *S. neurona* dispersal and that only a limited subset of possible parasite genotypes may have been introduced to recently established opossum populations. Moreover, our study confirms that diverse intermediate hosts share a common infection source, the opossum (*Didelphis virginiana*).

Published by Elsevier B.V.

Keywords: *Sarcocystis neurona*; Sea otter (*Enhydra lutris*); Raccoon (*Procyon lotor*); Skunk (*Mephitis mephitis*); Microsatellite; PCR

1. Introduction

Sarcocystis neurona was first recognized as an important cause of a neurologic disease in horses, equine protozoal myeloencephalitis (EPM) (Dubey et al., 2001a). More recently, its importance as a pathogen in sea otters has been established (Thomas et al., 2007). It also causes clinical sarcocystosis in cats, mink, raccoons, and other mammals. Opossums are its definitive hosts and other mammals act as intermediate or accidental hosts. Viable *S. neurona* has been isolated from sea otters, harbor seals, cats, opossums, raccoons, and horses (Dubey, 2000; Dubey et al., 1991, 2001b,c; Lindsay et al., 2000, 2001a; Mansfield et al., 2001; Miller et al., 2001a,b; Turay et al., 2002). The parasite is

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0304-4017/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetpar.2007.12.017

restricted to the Americas, coinciding with the geographic range of opossums (Dubey et al., 2001a).

Little genetic diversity has been described among isolates of *S. neurona* from its various hosts (Elsheikha et al., 2006; Elsheikha and Mansfield, 2007). One study using 12 highly polymorphic microsatellite markers (Asmundsson et al., 2006) found that *S. neurona* isolates from North America were derived from a single, intermixing population. By contrast, two South American parasite isolates were genetically distinct from *S. neurona* of North American origin (Asmundsson et al., 2006). Such markers can help resolve the historical and ongoing subdivision of biological populations because they are sampled from throughout the genome and because their alleles are presumed to be selectively neutral. Nonetheless, they sample only a portion of the genome and would not necessarily detect localized or recent changes to the genome. To further characterize the genetic structure of *S. neurona*, and to determine whether genetically distinct parasites are present in free-living wildlife populations, we amplified and characterized

Table 1
Description of *Sarcocystis neurona* isolates and genotypes

Host	ID	Geographic origin	Date Collected	Sn1	Sn2	Sn3	Sn4	Sn5	Sn6	Sn7	Sn8	Sn9	Sn10	Sn11
Raccoon	358	WI	01/05/2006	180	198	231	187	225	254	158	200	190	167	174
Raccoon	359	WI	01/07/2006	182	199	231	187	225	254	160	200	190	167	174
Raccoon	362	WI	01/08/2006	180	199	229	185	225	254	158	200	190	167	174
Raccoon	363	WI	01/08/2006	180	198	231	185	225	254	160	200	190	167	174
Raccoon	365	WI	01/08/2006	182	198	231	187	225	254	160	200	190	167	174
Raccoon	370	WI	01/08/2006	180	F	231	185	227	254	160	200	190	167	174
Raccoon	387	WI	01/14/2006	180	198	231	185	225	250	160	200	190	167	174
Raccoon	391	WI	01/25/2006	182	199	231	187	225	254	160	200	190	167	174
Raccoon	393	WI	01/27/2006	180	198	231	185	225	254	160	200	190	167	174
Raccoon	412	WI	01/31/2006	180	198	231	185	225	254	160	200	190	167	174
Raccoon	413	WI	02/06/2006	182	198	231	187	225	254	160	200	190	167	174
Skunk	365	WI	10/20/2005	180	199	231	187	225	250	160	200	190	167	174
Sea Otter	16227	Monterey, CA	04/1999	184	197	231	187	225	246	162	200	190	165	174
Sea Otter	19030	WA	04/2004	180	198	231	187	227	252	164	200	190	167	174
Sea Otter	16445	Santa Cruz, CA	10/1999	182	196	229	187	225	252	166	200	190	165	172
Sea Otter	15821	Santa Cruz, CA	06/1998	184	196	233	187	225	250	162	200	199	169	174
Sea Otter	16904	WA	07/2000	180	199	231	187	225	252	F	200	190	167	174
Sea Otter	19057	WA	05/2004	180	199	231	187	225	252	162	200	190	167	172
Sea Otter	18096	Monterey, CA	03/2002	F	196	233	185	225	250	158	200	190	169	174
Sea Otter	15822	Santa Cruz, CA	06/1998	184	196	233	185	227	250	158	200	199	169	174
Sea Otter	15713	WA	03/1998	184	199	231	187	225	252	162	200	190	167	172
Sea Otter	15792	Santa Cruz, CA	05/1998	184	196	233	185	225	250	164	200	199	169	174
Sea Otter	14675	Santa Cruz, CA	01/1997	184	197	229	187	227	252	158	200	190	165	174
Sea Otter	13631	San Luis Obispo, CA	05/1995	F	196	231	F	227	250	F	200	196	165	174
Sea Otter	14414	San Luis Obispo, CA	08/1996	188	199	231	187	225	250	168	200	196	165	172
Sea Otter	11429	Santa Cruz, CA	03/1993	182	196	229	187	225	252	164	200	190	165	174
Sea Otter	11450	San Luis Obispo, CA	04/1993	182	199	231	187	227	242	162	200	190	F	174
Sea Otter	12749	Santa Cruz, CA	04/1994	184	197	231	185	225	250	158	200	199	169	174
Sea Otter	12712	Santa Cruz, CA	04/1994	184	197	231	185	227	250	158	200	199	169	174
Sea Otter	10696	San Luis Obispo, CA	04/1992	F	F	233	185	227	244	F	200	190	F	174
Sea Otter	13502	WA	03/1995	182	197	229	191	225	242	162	200	190	165	174
Sea Otter	13590	San Luis Obispo, CA	04/1995	F	199	229	187	225	F	168	200	196	165	F
Sea Otter	14226	San Luis Obispo, CA	04/1996	184	197	231	185	225	250	158	200	199	169	174
Sea Otter	13479	San Luis Obispo, CA	03/1995	F	198	231	187	227	250	168	200	196	165	F

The columns of each locus (Sn1–Sn11) indicate the estimated number of base pairs amplified. *F* = amplification failure.

microsatellite loci from 22 infected sea otters, 11 raccoons, and 1 skunk.

2. Materials and methods

2.1. Isolates from Washington and California sea otters

The frozen brains of sea otters from Washington and California were shipped on ice from Department of Interior, United States Geological Survey, National Wildlife Health Center, WI (Table 1) to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, MD for genetic characterization of *S. neurona*.

2.2. Isolates from raccoons and skunk

Unfixed tongues from 47 trapped or road-killed raccoons and 1 skunk collected from a 350 km^2 area in Dane and Iowa counties (43′05″N and 89′50″W) of south-central Wisconsin from October 2005 to March 2006 were also sent to APDL for protozoal examination (Dubey et al., 2007). The muscle portion of the tongue was dissected and squash smears were examined microscopically for the presence of sarcocysts. Tissues infected with sarcocysts (11 raccoons and 1 skunk) were kept frozen at −20 °C until DNA extraction (Table 1).

2.3. Comparison isolates

Data derived from the new isolates were compared to a group of isolates detailed previously (Asmundsson et al., 2006) including nine Virginia opossums (*Didelphis virginiana*) and four horses (*Equus caballus*) from various US localities, one sea otter (*Enhydra lutris*) from Monterey Bay, CA, and two Brazilian opossums (*Didelphis albiventris*) from the vicinity of São Paolo.

2.4. Microsatellite analysis of S. neurona

DNA was extracted from the brain tissue of the sea otters, and from the tongues of the raccoons and skunk, using the DNeasy Tissue Kit (Qiagen). Negative control extractions were conducted in parallel to verify the absence of contaminating DNA. Microsatellite analysis and genotyping was performed as previously described (Asmundssson and Rosenthal, 2006) with the following modifications. Inconsistent amplification of markers Sn5 and Sn11 in some sea otter isolates motivated us to develop semi-nested polymerase chain reactions that provided for more robust amplification of these loci from these templates. Thus, a new primer (Sn5EF-GTGGAATGACGATGACAAGGA) was used in conjunction with primer Sn5R for 25 cycles of primary PCR using an annealing temperature of 55 °C. Upon completion, 1.5 μl of this reaction was used as template in the previously described PCR assay after the primary reaction was diluted 1:1 in water. To amplify marker Sn11, a new reverse primer (Sn11ER-GTTGTAACGG-CATTTGGGGT) was used in conjunction with primer Sn11F for 25 cycles of primary PCR with an annealing temperature of 60 °C. As above, this template was subsequently used in a secondary PCR according to previously described procedures. Genotyping the parasites from skunk and raccoon infections did not require nested PCR. Attempts to amplify marker SN12 often failed entirely. Thus, we restricted our analysis to markers Sn1–Sn11.

2.5. Clustering analysis

We subjected our entire dataset, comprising 34 new and 26 previously described isolates of *S. neurona* to an assignment procedure employing Bayesian statistics to explore how extensively *S. neurona* populations may be subdivided among their hosts or across their geographic range (Structure 2.1; Pritchard et al., 2000). This approach assigns individuals to one of 'K' populations based solely on its genotype (that is, without reference to *a priori* assumptions based on host or geography). An iterative procedure is used to minimize the genetic diversity within each population (each designated by an arbitrary color) and maximize the genetic distance between each population. The height of each bar indicates the confidence with which each individual can be assigned to any given population. A model assuming population admixture was implemented using one million MCMC replications and a burn-in period of 100,000 generations (Pritchand et al., 2000). New isolates of 22 sea otters, 11 raccoons, and 1 skunk were analyzed together with previously described isolates from 21 opossums, 4 horses, and 1 sea otter. To evaluate into how many populations the data might represent, we assessed the statistical fit assuming 2–9 population subdivisions (and depict representative results for 3–6 populations in Fig. 1).

2.6. Distance tree reconstruction

To quantify the extent of genetic distance among any such population subdivisions, we reconstructed a Neighbor Joining tree based on allele sharing distance

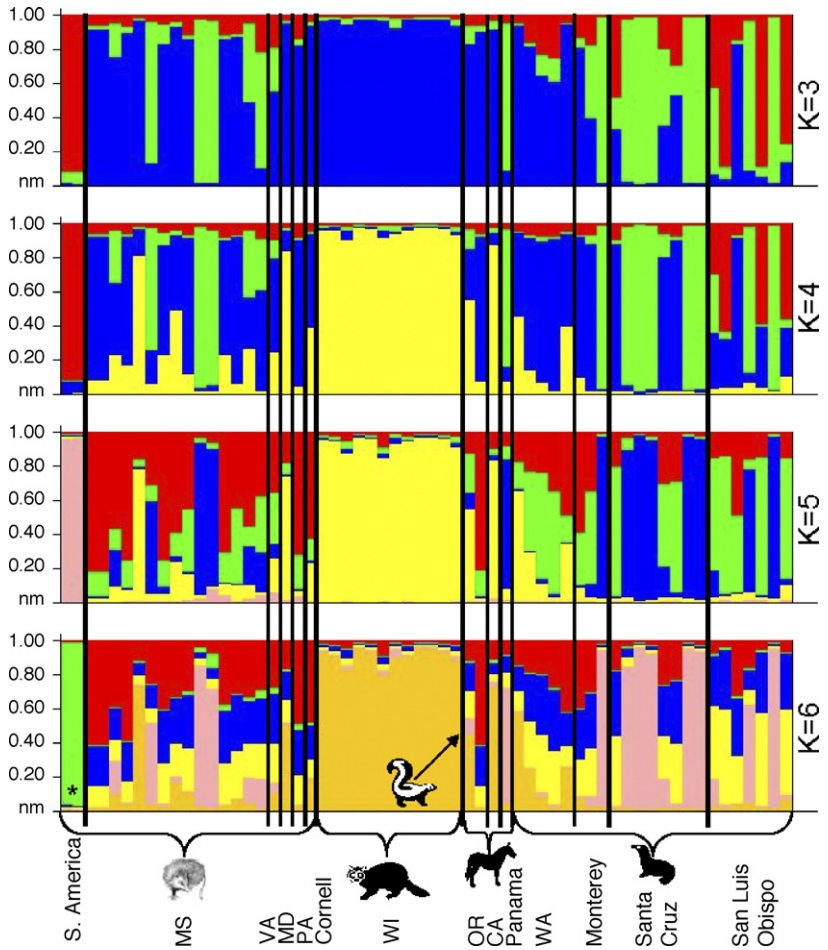

Fig. 1. Assignment of individual isolates to sub-populations. Arbitrary colors are assigned represent each of *K* pre-specified populations, and the height of each bar represent the confidence with which each individual can be assigned to that population based on microsatellite alleles. In each analysis, specimens from Wisconsin and South America are identified as genetically distinct. *South American isolates were from *Didelphis albiventris*.

(Goldstein and Pollock, 1997) among all North American isolates of *S. neurona*, rooted with two isolates derived from São Paolo, Brazil using populations v. 1.2.30. This distance represents the squared difference in allele size, summed across loci, between isolate pairs.

3. Results

After increasing the robustness of amplification for markers Sn5 and Sn11 by means of nested PCR, genotypes were obtained for more than 95% of isolates from 34 new specimens at 11 loci (Table 1). The observed genotypes best fit a model assuming the existence of four population subdivisions (Fig. 1). Under this assumption, the genetic distance among populations, and the genetic coherence within populations, was maximized. One of these four subpopulations (yellow bars when $K = 4$) was exclusively comprised of isolates from Wisconsin, representing infections in 11 raccoons and 1 skunk. Likewise, specimens from South America, identified as *S. neurona* on the basis of bioassays were assigned to a distinct population in all analyses (red bars when $K = 4$). Though genetically variable, other North American specimens from opossums, sea otters and horses were apportioned, with varying certainty, to the same pair of populations (blue and red bars, when $K = 4$).

Additional iterations of the analysis also recognized South American and Wisconsin isolates as distinct. Similarly, isolates from the terrestrial and marine mammals from other North American locales were genetically heterogeneous, irrespective of the degree of subdivision. Under the most statistically justified

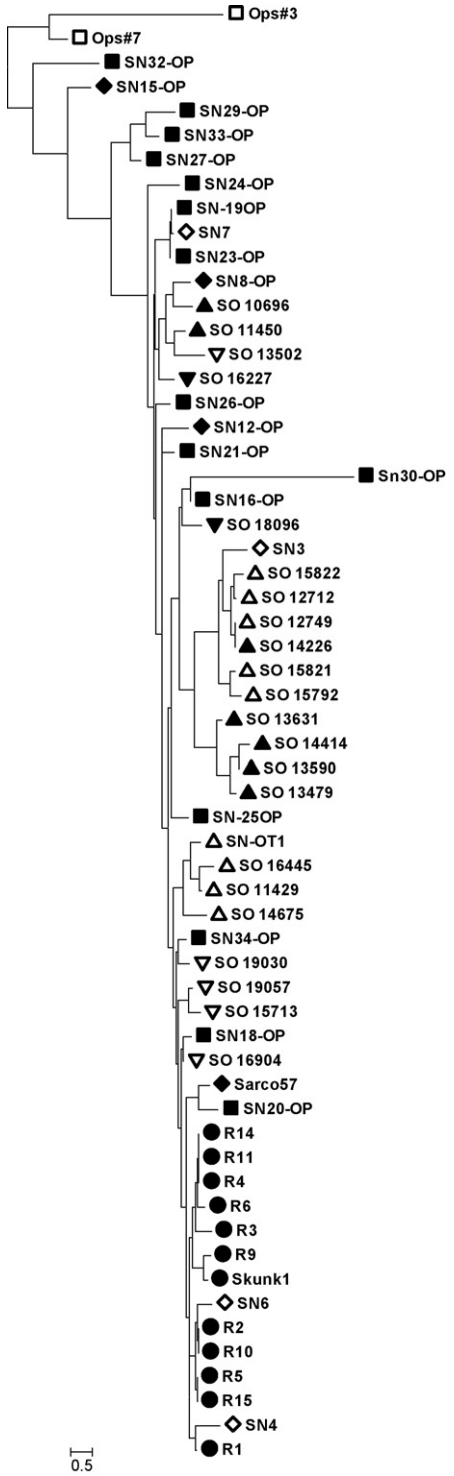

Fig. 2. Neighbor Joining tree based on allele sharing distance among all North American isolates of *Sarcocystis neurona*, rooted with two isolates derived from Sao Paolo, Brazil using populations v. 1.2.30. Isolates from WI (●) form a homogeneous group, as do certain other isolates designated by their origins (▲ and △ represent sea otter isolates from San Luis Obispo and Santa Cruz, CA, respectively). Comparatively diverse isolates from opossums in MS (■) occur throughout the tree. (◆) represents isolates from opossums from VA, MD, PA and Cornell. (▼) and (▽) represent sea otter isolates from Monterey, CA and WA, respectively, (◇) represents isolates from horses and (□) represents *S. neurona* isolates from South America.

assumptions, these isolates from sea otters and opossum were attributed to the same populations. We noted, however, that when five or more populations were specified, some genetic differentiation was implied between certain parasites isolated from marine mammals and many isolates from terrestrial mammals.

To examine how extensively groups of North American isolates of *S. neurona* differed from one another, a Neighbor Joining tree was reconstructed from the summed difference in allele size among pairs of isolates. The parasites of various North American locales differed far less from each other than from isolates derived from São Paolo, Brazil (Fig. 2). Nonetheless, associated specimens tended to resemble one another genetically (Fig. 2). For example, a clade of specimens almost exclusively comprised of sea otter isolates was further bifurcated into a lineage exclusively comprised isolates from San Luis Obispo, CA (▲, Fig. 2) and one encompassing a disproportionate number of isolates from Santa Cruz, CA (△). Similarly, isolates from the skunk and raccoons of WI (●) were only poorly differentiated from one another. Notably, specimens from opossums (■) in MS, (◆) in the Eastern US were interleaved with isolates from these various intermediate hosts, providing evidence that sea otters, horses (◇), skunks, and raccoons are exposed to a spectrum of sporocysts excreted by opossums. Sea otter isolates from WA (▽) exhibited genotypes similar to those evident in geographically disparate terrestrial mammals.

4. Discussion

Here, we have genetically characterized the natural infections of *S. neurona* in raccoons and skunk for the first time. Raccoons may play an especially important role in sustaining *S. neurona* transmission because they are abundant and frequently infected. Antibodies to *S. neurona* were found in 92.3% of 469 raccoons from VA (Hancock et al., 2004), 58.6% of 99 raccoons from FL, NJ, PA, and MA (Lindsay et al., 2001a,b), and 46% of 24 skunks and 100% of 12 raccoons from Connecticut (Mitchell et al., 2002). Viable *S. neurona* (designated SN-37R) was isolated by feeding muscles from naturally infected raccoons to laboratory-raised opossums

(Dubey et al., 2001b; Sofaly et al., 2002). Clinical EPM has been induced in horses by feeding sporocysts of this raccoon-derived SN37-R isolate of *S. neurona* (Sofaly et al., 2002; Saville et al., 2004) and tissue cysts were subsequently identified in raccoons (Stanek et al., 2002). Cheadle et al. (2001) demonstrated that skunk can act as an experimental intermediate host for *S. neurona*. Sarcocysts morphologically similar to those of *S. neurona* were found in one naturally infected skunk (Dubey et al., 2002) and schizonts reactive to *S. neurona*-specific antibodies were found in the brain of an encephalitic skunk (Dubey et al., 1996; Dubey and Hamir, 2000). In the present study, identification of *S. neurona* in raccoons and the skunk from WI was based solely on the DNA characterization.

Our study compared the distribution of genetic variation among 34 new isolates of *S. neurona* with 26 previously reported isolates. The earlier isolates included those from geographically widely dispersed opossums, including species endemic to South America, several isolates from horses, and one from a sea otter originating in Monterey, CA.

By broadening the scope of specimens considered in a comparative population genetic analysis, we have identified genetic cohesion among geographically proximate isolates of *S. neurona*. Genetic similarity characterizes the skunk and raccoon isolates from a small region of Wisconsin. Similarly, a genetically cohesive group was identified among many isolates from sea otters in Santa Cruz and San Luis Obispo, CA. However, sea otter isolates from Washington do not share genetic similarities with that group. Most of these (SO 19030, 19057, 15713 and 16904) resemble isolates from geographically disparate terrestrial mammals, whereas one (SO 13502) most closely resembles a sea otter isolate originating in San Luis Obispo, CA. Previous results identified geographic barriers to parasite gene flow only at a continental scale (Asmundsson et al., 2006) but these results affirm that limited dispersal can differentiate, at least to some modest extent, local *S. neurona* populations. Interestingly, *D. virginiana* became established in the Pacific coast as recently as the early 20th century, and the northern limit of its distribution occurred at the southern edge of Wisconsin as recently as 1926 (Chapman and Feldhamer, 1982). In contrast, far greater diversity is evident among the parasites of locales in which opossums have been endemic for far longer, such as Mississippi. Therefore, recently established opossum populations may have introduced parasite genotypes.

Such geographic associations appear to be temporally persistent. We cannot know, with certainty, when sea otters acquired their infections. However, no chronic infection state is known for these hosts. If fatal illness typically ensues from recent exposure, then one genetically distinguishable lineage of *S. neurona* may have been maintained in the vicinity of Santa Cruz, CA during a 4-year period (1994–1998) while a second lineage was present some 240 km to the Southeast in the vicinity of San Luis Obispo, CA (1995–1996). Additional sampling of opossums endemic to the regions would be needed to determine if these are contaminating the coastal environment with parasites with specific genotypes. And continued monitoring of sea otter populations would enable us to understand how long particular parasite lineages persist in a given habitat.

The genetic similarity of other sea otter isolates to those derived from terrestrial mammals reinforces an epidemiological connection between transmission on land and in coastal waters. This connection is supported by analyses conducted under the most statistically plausible models of population structure. It remains possible that migration barriers, or the substantial differences between terrestrial and marine environments, may modestly affect genotypic frequencies of parasites inhabiting coastal waters.

Certain methodological limitations might influence the extent to which true or artificial differences among parasite populations are recognized. On the one hand, actual barriers to gene flow will not be recognized in the event that these are too recent, or too ephemeral, to produce marked differences in the local abundance of particular alleles. On the other hand, artificial differences among localized populations can conceivably arise solely through sampling variance, although these would not be expected to produce strong geographic patterns.

Either local adaptation or random genetic drift can engender differences in the local abundance of particular microsatellite alleles. Adaptation can produce such outcomes by disfavoring the progeny of parents specialized in dissimilar ways, whereas genetic drift results when immigration is too infrequent to counteract the local loss of alleles. Either process could have contributed to the subtle differentiation identified between localized parasite populations. Nonetheless, the overall magnitude of such differences is slight.

In addition to these considerations, we believe it important to acknowledge the possibility that slight, systematic biases can be introduced when different researchers score microsatellite genotypes. In our experience, certain individuals can be difficult to score at certain loci, leading to some subjectivity in

genotyping. Such uncertainties should degrade 'true' evolutionary signal when a given dataset is evaluated by a person blinded to the origin of each sample, but can amplify the apparent differences between datasets scored by different researchers, as was the case here. We endeavored to mitigate any such effects by testing the agreement among repeated genotyping calls. Nonetheless, the subtle effects of slight differences in genotyping procedures could contribute differentiation between the parasites of terrestrial and marine mammals in this study, and should be borne in mind as additional specimens are characterized in the future.

Identifying strong and persistent genetic differences among the parasites of diverse terrestrial and marine mammals might have provided a basis for recognizing them as distinct species. However, these data suggest that although the dispersal of *S. neurona* encounters geographic barriers, the fatal encephalitis in southern sea otters should be attributed to the same etiological agent, excreted by opossums, responsible for EPM in horses and neurological disease in other terrestrial mammals.

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